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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12N 15/57, 9/54, C11D 3/386 — A1

(11) International Publication Number: WO 94/02618

(43) International Publication Date: 3 February 1994 (03.02.94)

(21) International Application Number: PCT/EP93/01917

(22) International Filing Date: 19 July 1993 (19.07.93)

(30) Priority data:
92202215.7 17 July 1992 (17.07.92) EP

(34) Countries for which the regional or international application was filed: NL et al.

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(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, US, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: HIGH ALKALINE SERINE PROTEASES

(57) Abstract

New PB92 or Subtilisin 309 mutant serine proteases are provided having specific mutations, resulting in a surprisingly better wash performance or in an improved storage stability with at similar or even better wash performance. These PB92 or Subtilisin 309 mutants include mutations at positions 60, 87, 97, 99, 102, 116, 117, 126, 127, 128, 130, 133, 134, 154, 156, 158, 159, 160, 164, 169, 175, 180, 182, 193, 197, 198, 203, 211, and 216. The new proteases, therefore, are very suitable for use in various types of detergents, whether or not in conjunction with other enzymes, for example amylases, cellulases and lipases. Preferred embodiments are the PB92 and Subtilisin 309 mutants having a mutation at position 102 and in particular those having at least one further mutation.

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HIGH ALKALINE SERINE PROTEASES

INTRODUCTION

Technical Field

The present invention relates to new high alkaline serine protease mutants having improved properties for use in detergents. These properties include improved stain removing ability in laundry detergent washing compositions, improved stain removing ability at low laundering temperature, improved stability in laundry detergents upon storage and improved stability in suds prepared from the detergents.

Background of the invention

Use of enzymatic additives, in particular proteolytic enzymes, in detergent compositions to enable removal of protein based soilings has been amply documented. See for example the published European Patent Applications EP-A-0220921 and EP-A-0232269, U.S. Patents Nos. 4,480,037 and Re 30,602, and the article "Production of Microbial Enzymes", Microbial Technology, vol. 1 (1979) 281-311, Academic Press.

Detergent compositions, which are applied for hard surface cleaning, toilet cleaning, dish washing and laundry cleaning, may be in a powder, liquid or paste form. Laundry detergents are generally divided into two major types, liquids and powders.

Proteolytic enzymes are generally difficult to combine with detergent compositions. They must be stable and active during application, for example in removing proteinaceous stains from textile during washing at temperatures ranging from about 10°C to over 60°C. Furthermore they must be stable for prolonged periods of time during storage in the detergent product. Consequently, enzymes have to be stable and functional in the presence of sequestering agents, surfactants, high alkalinity, often bleaching agents, and elevated temperature.

As there exist neither universal laundry detergents nor universal washing conditions (pH, temperature, sud-concentration, water hardness) that are used all over the world, the demands on enzymes may vary based on the type of detergent in which they are used and on the washing conditions.

A commercially important group of proteases is that of the so-called high alkaline proteases, derived from alkalophilic Bacilli. The commercially available high alkaline protease product MAXACAL® (Gist-brocades/IBIS) contains the serine protease "PB92", derived from Bacillus novo sp. PB92 (see U.S. Patent Re. No. 34,602). Its amino acid sequence is disclosed in EP-A-0283075 and EP-A-0284126. Also SAVINASE® (Novo-Nordisk) is a member of this group. SAVINASE contains the "Subtilisin 309" enzyme, which is derived from Bacillus strain NCIB 10147 (U.S. Patent No. 3,723,750). Its amino acid sequence is disclosed in WO 89/06279, where the strain is referred to as Bacillus lentus. The amino acid sequences of these two proteases appear to differ only at position 85 (taking the residue numbering of the PB92 protease, which corresponds to position 87 in the BPN' numbering), where PB92 has an asparagine ("N") in the one letter amino acid code) and "Subtilisin 309" a serine ("S").

Since the PB92 protease is active in stain removing at alkaline pH-values, it is commonly used as a detergent additive, together with detergent ingredients such as surfactants, builders and oxidizing agents. The latter agents are mostly used in powder form. The detergent additive may also contain other enzymes, for example amylases, cellulases and/or lipases, as far as they are compatible with the protease. PB92 protease has a high stain removing efficiency as compared to other proteases, such as the "classic" subtilisins which are well known in the art. This means that less PB92 protease is needed to obtain the same wash performance. Sensitivity to oxidation is an important drawback of the PB92 protease and all other known serine proteases used for application in detergents.

Originally the commercially available alkaline proteases such as MAXACAL® were developed for application in

detergents at enhanced temperatures in the range 40-60°C. However nowadays, because the growing emphasis on ecomomy, there is an ongoing tendency to switch to lower temperatures. As a consequence the lower wash performance at reduced temperatures, e.g. 15-25°C, is an important handicap of the excisting commercially alkaline proteases.

There are several ways of obtaining new enzymes for an intended application, which are all known to the skilled artisan. Modification of existing enzymes by protein engineering is likely to be the most popular and effective method nowadays.

The most specific way of obtaining modified enzymes is by site-directed mutagenesis, enabling specific substitution of one or more amino acids by any other desired amino acid. EP-A-0130756 exemplifies the use of this technique for generating mutant protease genes which can be expressed to give modified proteolytic enzymes. A very effective method is the oligo-nucleotide mediated site-directed mutagenesis, which allows a number of different mutations to be introduced at a specific part of a DNA sequence by using a single synthetic oligo-nucleotide preparation.

For a comprehensive summary of the various detergent compositions and enzymes, their physical forms, the conditions which the enzymes have to meet for optimal functioning, the problems and limitations of the currently available enzymes for use in detergent enzyme compositions, preparation and screening of mutant proteases, etc., reference may be made to EP-A-0328229, which is incorporated herein by reference.

WO 89/06279 claims inter alia mutants of the "Subtilisin 309" protease, in which one or more residues at the following positions are substituted (taking the original BPN' residue numbering): 6, 9, 11-12, 19, 25, 36-38, 53-59, 67, 71, 89, 104, 111, 115, 120, 121-122, 124, 128, 131, 140, 153-166, 168, 169-170, 172, 175, 180, 182, 186, 187, 191, 194, 195, 199, 218, 219, 222, 226, 234-238, 241, 260-262, 265, 268, or 275. The number of examples in this reference describing mutants which have been actually made and tested is restricted to only eight, while no more than four positions are involved. These

mutants are: S153A, G195D, G195E, N218S, [G195E M222A], [G195E M222C], M222A, and M222C.

EPA-A-0328229 discloses and claims inter alia mutant proteases which have at least 70% homology with the amino acid sequence of PB92 serine protease and differ by at least one amino acid residue at a selected site corresponding to 32, 33, 48-54, 58-62, 94-107, 116-118, 123-134, 150, 152-156, 158-161, 164, 166, 169, 175-186, 197, 198 and 203-216, 235, 243 and 259 in said PB92 serine protease, and have improved wash performance and/or improved stability relative to said PB92 serine protease. This reference is exemplified by 69 mutants, in which 17 positions are involved.

Despite the progress which seems to have been made in the past few years, there is a continuing interest in the development of new proteolytic enzymes with improved properties which make them more attractive for use in detergents. These properties may include, but are not limited to, better wash performance, improved stain removing ability at low laundering temperature, improved stability upon storage, or improved stability while they are used.

SUMMARY OF THE INVENTION

In one aspect the present invention provides new PB92 or Subtilisin 309 mutant serine protease having specific mutations, resulting in considerably improved properties which make them very suitable for application in detergents, especially laundry detergents. These PB92 or Subtilisin 309 mutants include mutations at positions 60, 87, 97, 99, 102, 116, 117, 126, 127, 128, 130, 133, 134, 154, 156, 158, 159, 160, 164, 166, 169, 175, 180, 182, 193, 197, 198, 203, 211, 212, and 216.

In a preferred embodiment of the invention there are provided PB92 and Subtilisin 309 mutants having a mutation at position 102, preferably in combination with at least one further mutation. Of these, the PB92 mutants [S99G,V102N] and [V102N,N198G] are most preferred.

In another aspect the invention provides new enzymatic

detergent compositions, comprising a proteolytic enzyme product which contains at least one of such new mutant proteolytic enzyme, whether or not in conjuction with other enzymes, for example amylases, cellulases and lipases.

These and other aspects of the invention will be further outlined in the detailed description hereinafter.

DETAILED DESCRIPTION OF THE INVENTION

By the term "improved properties" as used in this specification in connection with "mutant proteases" we mean proteolytic enzymes with improved wash performance or improved stability with retained wash performance, relative to the corresponding wild-type protease.

The term "wash performance" of mutant proteases is defined in this specification as the contribution of a mutant protease to laundry cleaning additional to the effect of the detergent composition without enzyme under relevant washing conditions.

The term "relevant washing conditions" is used to indicate the conditions, particularly washing temperature, time, washing mechanics, sud concentration, type of detergent and water hardness, actually used in households in a detergent market segment.

The term "improved wash performance" is used to indicate that the wash performance of a mutant protease, on weight basis, is at least greater than 100% relative to the corresponding wild-type protease under relevant washing conditions.

The term "retained wash performance" is used to indicate that the wash performance of a mutant protease, on weight basis, is at least 80% relative to the corresponding wild-type protease under relevant washing conditions.

The term "improved stability" is used to indicate better stability of mutant proteolytic enzymes in laundry detergents during storage and/or their stability in the sud, which includes stability against oxidizing agents, sequestering agents, autolysis, surfactants and high alkalinity, relative to

the corresponding wild-type enzyme.

EP-A-0328229 describes a method in which the preparation of mutant proteases is combined with an efficient selection procedure on the performance of these proteases. The test system is based on the removal of protease sensitive stains from test swatches in a launderometer or tergotometer, imitating relevant washing conditions. Suitable test swatches are, for example, the commercially available EMPA swatches. (Eidgenössische Material Prüfungs und Versuch Anstalt, St. Gallen, Switzerland) artificially soiled with proteinaceous stains. Relevant stains on swatches for testing proteases include blood, grass, chocolate, and other proteinaceous stains. The reference also discloses that in this test system other relevant factors, such as detergent composition, sud concentration, water hardness, washing mechanics, time, pH and temperature, are controlled in such a way that conditions typical for household application in a certain market segment can be imitated.

Wash performance of proteases is conveniently measured by their ability to remove certain representative stains under appropriate test conditions. This ability can be suitably determined by reflectance measurements on the test cloths, after washing with and without enzymes in a launderometer or tergotometer. The laboratory application test system according to the invention is representative for household application when used on proteases which are modified by DNA mutagenesis.

In order to practice the present invention essentially the same method can be used for the preparation, screening and selection of further mutant enzymes derived from wild-type enzymes which are produced by alkalophilic Bacilli. Preferred mutants are those encoded by a gene derived from a wild-type gene encoding the PB92 serine protease or the Subtilisin 309 serine protease and which show improved properties under the test conditions mentioned above. Also genes encoding closely related serine proteases, preferably having a homology greater than about 70%, more particularly greater than about 90%, are very suitable.

It will be clear that either oligonucleotide aided

site directed mutagenesis or region directed random mutagenesis can be used or any other suitable method for efficiently generating mutations in the protease gene of choice.

In accordance with the invention, various mutants were obtained with unexpectedly improved properties, i.e. a considerably higher wash performance, improved stain removing ability at low laundering temperature, or considerably improved storage stability with a similar or even better wash performance. These improvements were surprising, since they were neither suggested by, nor could they be derived in any way from the teaching of EP-A-328229 or any other prior art, either alone or when taken together.

The present invention therefore provides a mutant protease for use in detergents which comprises:

having at least 70% homology with either the amino acid sequence of PB92 serine protease having the amino acid sequence:

H_2N-A-Q-S-V-P-W-G-I-S-R-V-Q-A-P-A-A-H-N-R-G-L-T-G-S-G-V-K-V-A-
V-L-D-T-G-I-S-T-H-P-D-L-N-I-R-G-G-A-S-F-V-P-G-E-P-S-T-Q-D-G-N-
G-H-G-T-H-V-A-G-T-I-A-A-L-N-N-S-I-G-V-L-G-V-A-P-N-A-E-L-Y-A-V-
K-V-L-G-A-S-G-S-G-S-V-S-S-I-A-Q-G-L-E-W-A-G-N-N-G-M-H-V-A-N-L-
S-L-G-S-P-S-P-S-A-T-L-E-Q-A-V-N-S-A-T-S-R-G-V-L-V-V-A-A-S-G-N-
S-G-A-G-S-I-S-Y-P-A-R-Y-A-N-A-M-A-V-G-A-T-D-Q-N-N-N-R-A-S-F-S-
Q-Y-G-A-G-L-D-I-V-A-P-G-V-N-V-Q-S-T-Y-P-G-S-T-Y-A-S-L-N-G-T-S-
M-A-T-P-H-V-A-G-A-A-A-L-V-K-Q-K-N-P-S-W-S-N-V-Q-I-R-N-H-L-K-N-
T-A-T-S-L-G-S-T-N-L-Y-G-S-G-L-V-N-A-E-A-A-T-R-COOH;

or the amino acid sequence of Subtilisin 309 serine protease having the amino acid sequence:

H_2N-A-Q-S-V-P-W-G-I-S-R-V-Q-A-P-A-A-H-N-R-G-L-T-G-S-G-V-K-V-A-
V-L-D-T-G-I-S-T-H-P-D-L-N-I-R-G-G-A-S-F-V-P-G-E-P-S-T-Q-D-G-N-
G-H-G-T-H-V-A-G-T-I-A-A-L-N-N-S-I-G-V-L-G-V-A-P-S-A-E-L-Y-A-V-
K-V-L-G-A-S-G-S-G-S-V-S-S-I-A-Q-G-L-E-W-A-G-N-N-G-M-H-V-A-N-L-
S-L-G-S-P-S-P-S-A-T-L-E-Q-A-V-N-S-A-T-S-R-G-V-L-V-V-A-A-S-G-N-
S-G-A-G-S-I-S-Y-P-A-R-Y-A-N-A-M-A-V-G-A-T-D-Q-N-N-N-R-A-S-F-S-
Q-Y-G-A-G-L-D-I-V-A-P-G-V-N-V-Q-S-T-Y-P-G-S-T-Y-A-S-L-N-G-T-S-
M-A-T-P-H-V-A-G-A-A-A-L-V-K-Q-K-N-P-S-W-S-N-V-Q-I-R-N-H-L-K-N-
T-A-T-S-L-G-S-T-N-L-Y-G-S-G-L-V-N-A-E-A-A-T-R-COOH;

differing by at least one amino acid residue at a

selected site corresponding to positions positions 60, 87, 97, 99, 102, 116, 117, 126, 127, 128, 130, 133, 134, 154, 156, 158, 159, 160, 164, 169, 175, 180, 182, 193, 197, 198, 203, 211, and 216 in said PB92 serine protease or said Subtilisin 309 serine protease,

having improved wash performance and/or improved stability relative to said PB92 serine protease or said Subtilisin 309 serine protease.

A preferred group of mutant protease according to the invention are those mutants of PB92 or Subtilisin 309 protease which differ by at least one of the following mutations: [N60E], [N60E,M216S], [E87Q], [E87S], [S97D], [S99G], [S99G, V102I], [S99G,V102L], [S99G,V102N], [S99G,S130G], [S99G, Y203W], [S99G,M216S], [S99T], [V102A], [V102A,M216S], [V102E], [V102G], [V102H], [V102I], [V102I,G116V,S126V,P127M], [V102I,G116V,S126V,P127M], [V102I,S130G], [V102L], [V102L, G116V,S126V,P127M], [V102L,S130G], [V102L,M216F], [V102L, M216S], [V102M], [V102N], [V102N,XYZ, where XYZ is any modified amino acid], [V102N,R164Y], [V102N,N198G], [V102N,N197T,N198G], [V102N,N198G,Y203W], [V102N,Y203W], [V102N,L211E], [V102N,M216X, where X is any amino acid except M], [V102N,M216S], [V102P], [V102P,M216S], [V102Q], [V102Q,M216S], [V102S], [V102S,M216S], [V102T], [V102Y], [G116V,S126L,P127N, S128V,A156E], [G116V,S126L,P127N,S128V,Y203W], [G116V,S126L, P127Q,S128A,S160D], [G116V,S126L,P127Q,S128A,M216S], [G116V, S126N,P127S,S128A], [G116V,S126N,P127S,S128A,M216Q], [G116V, S126N,P127S,S128A,M216S], [G116V,S126R,P127Q,S128D,M216S], [G116V,S126R,P127Q,S128D,M216S], [G116V,S126V,P127E,S128K, S160D], [G116V,S126V,P127M,S160D], [G116V,S126V,P127M,N198G], [G116V,S126V,P127M,Y203W], [G116V,S126V,P127M,Y203G], [M117L], [S126F,P127X, where X is any amino acid except P], [S126M, P127A,S128G,S160D], [S126M,P127A,S128G,M216Q], [S126V,P127M], [P127E], [P127E,S128T,M216S], [P127E,Y203W], [P127E,L211E], [S130G], [S130G,Y203W], [L133I], [L133M], [L133W], [L133Y], [E134C], [S154D,S160G], [S154G,S160G], [S154E], [S154G], [S154N], [A156D], [A156E], [A156G], [S158D], [S158E], [S158E, I159L], [S158N], [S159E,I158L], [S160D,A166D,M169I], [S160D, N212D], [S160D,M216Q], [S160E], [S160G], [R164I], [R164M],

[R164V], [R164Y], [D175E], [R180I], [V197L], [V197N], [V197T], [V197T,M216S], [V197W], [N198C], [N198D], [N198E], [N198G], [N198G,Y203W], [N198G,M216S], [N198Q], [N198S], [N198V], [Y203C], [Y203E], [Y203G], [Y203K], [Y203L], [Y203L,V193A], [Y203T], [Y203T,S182N], [Y203V], [Y203V,V193A], [Y203W], [Y203W,M216S], [L211E], [L211X,N212Z, where X is any amino acid except L and Z is any amino acid except N], [L211E,M216S], and [N212E];

having improved wash performance and/or improved stability relative to said PB92 serine protease or said Subtilisin 309 serine protease.

Preferably, the mutant proteases according to the present invention are in substantially pure form.

According to an aspect of the invention, certain new mutant proteases show a considerably improved resistance to oxidation, whereas their wash performance is also better and in many cases significantly better than the wash performance of the corresponding wild-type protease. These mutant enzymes have in common that the methionine ("M") at position 216 is substituted by another amino acid, preferably serine ("S") or glutamine ("Q"). Also substitution by phenylalanine ("F") or alanine ("A") is suitable. Further substitutions include the positions 60, 99, 102, 116, 127, 128, 130, 154, 156, 158, 197, 198, 203, 211 and 212. Preferred enzymes are those M216S and M216Q mutants which are further substituted at position 102 or at one or more of the positions 116, 126, 127 and 128. Also M216S and M216Q mutants with substitutions at positions 197, 198 and 203 are of particular interest. Preferred mutants are [N60E,M216S], [S99G,M216S], [V102A,M216S], [V102L,M216S], [V102N,M216S], [V102P,M216S], [V102Q,M216S], [V102S,M216S], [G116V,S126L,P127Q,S128A,M216S], [G116V,S126N,P127S,S128A, M216S], [G116V,S126R,P127Q,S128D,M216S], [P127E,S128T,M216S], [V197T,M216S], [N198G,M216S], [Y203W,M216S], [L211E,M216S], [G116V,S126N,P127S,S128A,M216Q], [S126M,P127A,S128G,M216Q], [V102L,M216F].

It should be noted that EP-A-0328229 describes improved oxidation stability with retained wash performance of certain M216S and M216Q mutants of PB92 and similar high alkaline

serine proteases. However this reference does not teach or suggest that the "216" mutants of PB92 or Subtilisin 309 with the above-defined mutations would result even in a significantly improved wash performance.

In another aspect of the invention certain new mutant proteases which are generally not oxidation resistant, show a considerably improved wash performance. These mutant enzymes have one or more substitutions at positions 87, 97, 99, 102, 116, 117, 126, 127, 128, 130, 133, 134, 154, 156, 158, 159, 160, 164, 166, 169, 175, 180, 182, 193, 197, 198, 203, 211 and 212. Preferred mutants are those which have at least two modifications out of these defined positions. These modifications include the positions: 99 combined with at least one additional mutation at a position selected from the group comprising positions 102, 130 or 203; 102 combined with at least one additional mutation at a position selected from the group comprising positions 87, 97, 116, 117, 126, 127, 128, 130, 133, 134, 154, 156, 158, 159, 160, 164, 166, 169, 175, 180, 182, 193, 197, 198, 203, 211 or 212, preferably with at least one additional mutation at a position selected from the group comprising positions 130, 164, 197, 198, 203 or 211; 116, 126, 127, 128 combined with at least one additional mutation at a position selected from the group comprising positions 99, 102, 130, 156, 160, 197, 198, 203, 211, 212, preferably with at least one additional mutation at a position selected from the group comprising positions 102, 156, 160, 198, 203, 211; 126 and 127, preferably with one additional mutation at a position selected from the group comprising positions 102, 156, 160, 198, 203 or 211; 130 and 203; 154 and 160; 158 and 159; 160,166 and 169; 160 and 212; 198 and 203; 203 and 182; 203 and 193; 211 and 212. Preferred mutants are [S99G,V102N], [S99G,V102L], [S99G,V102I], [S99G,S130G], [S99G,Y203W], [V102I,S130G], [V102L,S130G], [V102N,R164Y], [V102N,N198G], [V102N,N197T,N198G], [V102N,N198G,Y203W], [V102N,Y203W], [V102N,L211E], [V102I,G116V,S126V,P127M], [V102L,G116V,S126V,P127M], [G116V,S126L,P127Q,S128A,S160D], [G116V,S126L,P127N,S128V,A156E], [G116V,S126L,P127N,S128V, Y203W], [G116V,S126N,P127S,S128A], [G116V,S126V,P127E,S128K,

S160D], [G116V,S126V,P127M,S160D], [G116V,S126V,P127M,N198G], [G116V,S126V,P127M,Y203W], [G116V,S126V,P127M,Y203G], [S126M,P127A,S128G,S160D], [P127E,L211E], [P127E,Y203W], [S126F,P127A], [S126F,P127D], [S126F,P127H], [S126F,P127N], [S126F,P127Q], [S126V,P127M], [S130G,Y203W], [S154G,S160G], [S154D,S160G], [S158E,I159L], [S160D,A166D,M169I], [S160D, N212D], [N198G,Y203W], [Y203T,S182N], [Y203V,V193A], [Y203L, V193A], [L211G,N212D], [L211N,N212D], [L211V,N212D], [L211Y, N212S]

In still another aspect of the invention certain new mutant PB92 and Subtilisin 309 proteases exhibit unexpected activity on cacao stains, which was in no way predictable from the prior art. Such mutant proteases have one or more substitutions at positions 102, 116, 117, 126, 127, 128, 133, 154, 156, 158, 159, 160, 164, 197, 198, 203, 211 and 216. Preferred mutants are those which have at least two modifications out of these defined positions. These modifications include the positions : 102 combined with at least one additional mutation at a position selected from the group comprising positions 164 or 211; 127 combined with at least one additional mutation selected from the group comprising positions 203 or 211 ; 154 and 160 ; 158 and 159. In addition, these modifications include position M216S and M216Q combined with at least one additional mutation at positions 102 or 211. Preferred mutants are : [V102N,R164Y], [V102N,L211E], [V102N,N198G], [P127E,Y203W], [P127E,L211E], S154G,S160G], [S154D,S160G], [S158E,I159L], [M216S,V102Q], [M216S,L211E]. In addition preferred mutants are the PB92 M216S mutants with further substitutions V102Q and L211E.

In still a further aspect of the invention certain new mutant PB92 and Subtilisn 309 proteases exhibit improved stain removing ability at lower laundering temperatures, e.g. about 20°C. These mutants have usually one or more substitutions in the PB92 or Subtilisin 309 enzyme at position 99, 102, 116, 126, 127, 128, 130, 160, 197, 198 and 203. Preferred mutants are those which have at least two modifications out of these defined positions. These modifications include the positions: 99, combined with at least one additional mutation at positions

102 or 130, preferably with a mutation at position 130; 102 combined with at least one additional mutation selected from the group comprising positions 197, 198 or 203, preferably with at least one additional mutation at positions 99 or 198, most preferably with an additional mutation at position 99 or 198; 126 combined with at least one additional mutation at positions 116, 127, 128 or 160, preferably 126 combined with 127. Preferred mutants are [S99G,S130G], [S99G,V102N], [S99G,V102I], [V102N,N198G], [V102N,Y203W], [V102N,V197I,N198G], [S126V, P127M], [S126F,P127N], [G116V,S126V,P127M,S160D], [G116V,S126L, P127Q,S128A,S160D].

Useful mutants may also be made by combining any of the mutations or sets of mutations described in this specification. Besides, it is possible to combine useful mutations as disclosed herein with mutations at other sites, which may or may not cause a substantial change in the properties of the enzyme.

To illustrate the significance of the approach used in this invention for obtaining new proteases suited for application in laundry detergents, i.e. by using representative laundry application testing as primary selection criterion, the results of the wash performance tests of mutant PB92 proteases were compared with biochemical parameters as usually determined in protein biochemical and enzymological research. These results allow the conclusion that any relation between parameters determining affinity for defined substrates and kinetics of the proteolytic reaction and wash performance is absent.

Therefore, it is of course also possible to combine two or more mutants with different properties in one enzyme product or in the same washing process. Such combination may or may not have a synergistic effect.

The invention comprises also the use of one or more mutant proteolytic enzymes, as defined hereinbefore, in a detergent composition or in a washing process. Such detergent composition may also contain one or more other enzymes, for example an amylase, cellulase or lipase which should be compatible with the protease or proteases of choice. The

selection of the best combination of enzymes usually depends on the requirements and needs of the customer, but generally does not require inventive skill.

Finally, it will be clear that by deletions or insertions of the amino acids in the protease polypeptide chain, either created artificially by mutagenesis or naturally occurring in proteases homologous to PB92 protease or Subtilisin 309, the numbering of the amino acids may change. However, it is to be understood that positions homologous to amino acid positions of PB92 protease or Subtilisin 309 will fall under the scope of the claims.

The mutant proteases according to the invention can be made in essentially the same way as described in EP-A-0328229. Also, the preparation of the genes which encode the desired mutant proteases, the cloning and expression of said genes, the choice of a suitable host, the fermentation conditions, recovery, purification, screening and selection of the enzymes, etc., are essentially the same as described in EP-A-0328229 and are well within the skill of an ordinary worker.

The following Examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL SECTION.

Materials and Methods which includes construction of the mutants, production of the mutants, purification, high performance liquid chromatography (HPLC) using cation exchange resin and gel filtration column, polyacrylamide gel-electrophoresis, active-site titration and determination of the kinetic parameters are similar or identical to those described in EP-A-0328229, except when stated otherwise. The mutants which are marked in the examples with the extension $^{+DTT}$ were purified and stored in the presence of 2 mM dithiothreitol (DTT).

EXAMPLE 1

The wash performance of various PB92 protease mutants was determined in a specially developed washing test which is described in detail in EP-A-0328229. In addition to the sodium-tripolyphosphate (STPP) containing powder detergent IEC-STPP in this example also a non-phosphate containing powder detergent (IEC-zeolite) was used. The typical features of both test systems which were applied to test the wash performance of the new protease mutants are summarized below:

Washing system	IEC-STPP	IEC-zeolite
Dosed detergent/bleach	4 g/l	7 g/l
sud volume per beaker (ml)	250	200
temperature (°C)	40	30
time (min.)	30	30
detergent	IEC-STPP	IEC-zeolite
detergent dosage (g/l)	3.68	5.6
Na-perborate.4aq. (g/l)	0.32	1.4
TAED (mg/l)	60	210
EMPA 116 / 117 (5x5cm)	2 / 2	2 / 2
CFT AS-3 CACAO (5x5cm)	0	2
EMPA 221 clean swatch (10x10cm)	0	2
Stainless steel balls (ϕ6mm)	0	15
[Ca^{2+}] (mM)	2	2
[Mg^{2+}] (mM)	0.7	0.7
[$NaCO_3$] (mM)	2.5	0

The IEC-STPP detergent powder (IEC Test Detergent Type I, Formulation May 1976) and the IEC-zeolite detergent powder (Formulation April 1988) were purchased from WFK-Testgewebe GmbH, Adlerstraße 44, D-4150, Krefeld, Germany. The performance on cacao was measured on CFT AS-3 swatches (purchased from CFT, Center For Test Materials, PO Box 120, Vlaardingen, The Netherlands). Two mutants, E87S and E87Q, were tested in the IEC-STPP system at 10g/l of STPP/bleach containing powder detergent as indicated in Table II. In addition performance measurements at 4g/l were made in the IEC-STPP system which was

slightly modified (indicated as ADE+ in the tables): Instead of 40°C, 30 minutes and 2mM Ca^{2+}, the wash performance tests were carried out at 30°C during 20 minutes in the presence of 5mM Ca^{2+}. In addition 2 EMPA 221 swatches and 15 stainless steel balls with a 6 mm diameter were included.

The results are summarized in the accompanying Tables I, II, III .

EXAMPLE 2

In order to determine the wash performance of some of the new PB92 protease mutants under conditions of low detergency to mimic typically U.S. conditions, the wash performance was determined in a washing test similar to the test described in Example 1, but with some modifications. The main characteristics of the test are summarized below:

sud volume per beaker (ml)	200
time (min.)	20
detergent A dosage (g/l)	1.3
EMPA 116 / 117 (5x5cm)	2 / 2
CFT AS-3 cacao (5x5cm)	2
EMPA 221 clean swatch (10x10cm)	2
Stainless steel balls (ϕ6mm)	15
[Ca^{2+}] (mM)	2
[Mg^{2+}] (mM)	0.7

I. Oxidation resistant PB92 M216 protease mutants - wash performance ≥100%

Positions involved: 60, 99, 102, 116, 126, 127, 128, 197, 198, 203, 211

Protease mutant	STPP 4g/l %	zeolite 7g/l %	k_{cat} 1/s	K_m mM
PB92 protease (unmodified)	100	100	105	1.0
PB92 mutant with M216S and:				
N60E	120	76[117]	7	2.3
S99G		119	6	1.3
V102A		113	n.d.	n.d.
V102L		125	20	2.1
V102N		113	26	4.3
V102P		135	n.d.	n.d.
V102S		106	n.d.	n.d.
G116V,S126L,P127Q,S128A	100		23	8.7
G116V,S126N,P127S,S128A	110		7	4.4
G116V,S126R,P127Q,S128D	120		7	1.3
P127E,S128T	160	45	5	1.0
V197T		129	9	1.7
N198G		133	6	1.2
Y203W		132	13	1.8

I. Oxidation resistant PB92 M216 protease mutants - wash performance >100%

(Cont'd)

Protease mutant	STPP 4g/l %	zeolite 7g/l %	k_{cat} 1/s	K_m mM
PB92 mutant with **M216Q** and:				
G116V,S126N,P127S,S128A	130	70	3	4.5
S126M,P127A,S128G	100		36	5.1
PB92 mutant with **M216F** and:				
V102L		135	9	1.3

^{117} : Performance measured on EMPA 117.
n.d.: Not determined

II. Non-oxidation resistant PB92 protease mutants (WP>100%)

Positions involved: 87, 97, 99, 102, 116, 117, 126, 127, 128, 130, 133, 134, 154, 156, 158, 160, 164, 166, 169, 175, 180, 182, 193, 197, 198, 203, 211 and 217

Protease mutant	STPP 4g/l %	zeolite 7g/l %	k_{cat} 1/s	K_m mM
PB92 protease (unmodified)	100	100	105	1.0
E87S	140[10g/l]	126	134	1.7
E87Q	145[10g/l]	115[117]	100	1.3
S97D	160		35	0.4
S99G		170	63	0.5
S99G,V102I		226	202	1.2
S99G,V102L		209	166	1.0
S99G,V102N		213	296	2.4
S99G,S130G		190	64	1.2
S99G,Y203W		148	77	0.6
S99T		137	81	1.0
V102A		110[117]	23	0.3
V102G		111	217	0.5
V102H		106	78	0.6
V102I		180	252	1.3
V102I,G116V,S126V,P127M		182	206	2.5
V102I,S130G		180	141	2.0
V102L		180	194	0.8
V102L,G116V,S126V,P127M		147	160	2.3
V102L,S130G		154	159	1.7

II. Non-oxidation resistant PB92 protease mutants

(Cont'd)

Protease mutant	STPP 4g/l %	zeolite 7g/l %	k_{cat} 1/s	K_m mM
V102M		136	253	1.3
V102N		170	199	2.3
V102N,N198G		253	223	3.0
V102N,N197T,N198G		227	247	3.1
V102N,N198G,Y203W		162	210	2.3
V102N,Y203W		178	252	1.9
V102P		145	13	0.4
V102Q		150	87	1.0
V102S		136	47	0.4
V102T		165	109	0.9
V102Y		124	275	0.3
G116V,S126L,P127Q,S128A,S160D	200		65	9.1
G116V,S126L,P127N,S128V,Y203W		138	253	3.6
G116V,S126N,P127S,S128A	130		64	2.4
G116V,S126V,P127E,S128K,S160D	175		30	4.4
G116V,S126V,P127M,S160D	235		28	3.4
G116V,S126V,P127M,N198G		159	162	1.9
G116V,S126V,P127M,Y203W		132	186	1.4
G116V,S126V,P127M,Y203G		108	154	1.8
S126F,P127A	130		223	10.0
S126F,P127D	120		112	8.2
S126F,P127H	150		197	7.8
S126F,P127N	200		80	3.3
S126F,P127Q	150		104	5.0
S126M,P127A,S128G,S160D	300		200	1.9

II. Non-oxidation resistant PB92 protease mutants

(Cont'd)

Protease mutant	STPP 4g/l %	zeolite 7g/l %	k_{cat} 1/s	K_m mM
S126V,P127M		200	191	1.7
P127E	200	140	137	1.6
S130G		170	85	1.5
S130G,Y203W		142	65	1.2
L133W		125	274	1.5
L133Y		125	n.d.	5.9
E134C^{+DTT}		170	n.d.	n.d.
S154E	200^{ADE+}		36	1.0
S154G		110	70	0.9
S154N		133	79	1.1
A156D	195^{ADE+}	120	77	0.9
A156G		104	61	0.5
S158G		105	82	0.9
S158N		138	71	0.6
S160D,A166D,M169I	200	120	13	1.2
S160D,N212D	120		12	1.5
S160G	100	115^{117}	29	1.7
R164M		110	99	1.0
R164V		131	121	1.2
R164Y		135	115	0.8
D175E		113	99	0.9
R180I		120	106	0.9
S182N,Y203T		125	94	0.7
V193A,Y203L		132	85	0.6
V193A,Y203V		132	86	0.6
V197N		113	99	1.0

II. Non-oxidation resistant PB92 protease mutants

(Cont'd)

Protease mutant	STPP 4g/l %	zeolite 7g/l %	k_{cat} 1/s	K_m mM
V197T		120	146	1.1
V197W		115	62	0.9
N198C^{+DTT}		124	n.d.	n.d.
N198G		152	92	1.1
N198G,Y203W		132^{117}	82	0.7
N198S		125	84	0.7
N198V		121	104	0.8
Y203E		130	111	0.6
Y203G		135	91	1.1
Y203K		108	103	0.6
Y203L		106^{117}	132	0.6
Y203T		135	92	0.6
Y203V		135	90	0.6
Y203W		165	144	1.0
L211E		164	9	0.9
L211G,N212D		105	39	1.2
L211N,N212D		132	16	0.7
L211V,N212D		106	26	1.4
L211Y,N212S	123		81	0.5
N212E	140		94	1.2

^{117} : Performance measured on EMPA 117.
n.d.: Not determined

III. PB92 protease mutants and their performance in cacao

Positions involved: 102, 116, 117, 126, 127, 128, 133, 154, 156, 158, 159, 160, 164, 197, 198, 203, 211 and 216

PB92 protease mutant	Wash Performance zeolite at 7g/l(%)			Kinetic parameters	
	116	117	choc	K_{cat} 1/s	K_m mM
V102E	87		133	55	2.2
V102N,R164Y	108	87	124	247	2.8
V102N,L211E	101	80	142	48	2.8
G116V,S126L,P127N,S128V,A156E	108	73	118	170	2.5
M117L	126	120	147	64	0.7
P127E,Y203W	105	103	134	135	1.0
P127E,L211E	63	47	119	9	1.1
L133I	126		135	43	0.7
L133M	113		126	108	0.6
S154D,S160G	109		116	32	1.7
S154G,S160G	124		132	34	2.2
A156E	140	137	173	105	1.3
S158D	139	126	190	91	1.1
S158E	123	121	176	101	1.1
S158E,I159L	118	132	132	90	1.0
S160E	104	110	145	17	0.5
R164I	119	117	126	127	1.1
V197L	79	106	119	60	0.8
N198D	110	110	153	92	0.8
N198E	102	123	159	87	0.7
N198Q	100	111	110	64	0.7
Y203C+DTT	95	107	129	n.d.	n.d.

III. PB92 protease mutants and their performance on cacao

(Cont'd)

PB92 protease mutant	Wash Performance zeolite at 7g/l(%)			Kinetic parameters	
	116	117	choc	k_{cat} 1/s	K_m mM
PB92 mutant with M216S and:					
V102Q	96	87	106	n.d.	n.d.
L211E	100		127	2	1.1

116 : Performance measured on EMPA 116;
117 : Performance measured on EMPA 117.
choc : Performance measured on CFT AS-3
n.d.: Not determined

The composition of Detergent A was as follows:

ingredients	% by weight
alcohol ethoxylate	13%
LAS-90	7%
polyacrylate	1%
zeolite	35%
Na-silicate	3%
Na_2CO_3	20%
tri-Na-citrate.$2H_2O$	4%
Na_2SO_4	8%
water	to 100%

Prior to addition of PB92 protease or mutants thereof, the pH of the wash liquor was adjusted to 10.2. The results are shown in Table IV.

In addition the wash performance of some of the mutants was determined at lower temperature. The results at 20°C are shown in table IV. All the mutants which are shown perform significantly better at 20°C than does the wild type under these conditions. Very surprisingly some of the mutants, such as [V102N,S99G], [V102N], [G116V, S126V, P127M,S160D] do show a better wash performance at 20°C than at 30°C. This is opposite to what was expected from the behaviour of wild type PB92 : The wash performance of PB92 goes down upon lowering the laundering temperature. So it seems that our approach to improve the wash performance of an alkaline protease by site specific engineering can also shift the temperature at which these proteases exhibit optimal performance.

Table IV : Wash performance new PB92 mutants at different temperatures:

wash performance (%)		
PB92 protease mutants	temperature	
	30°C	20°C
S99G	123	n.d.
S99G, S130G	188	173[117]
V102I, S99G	117[117]	n.d.
V102N, S99G	163	181
V102N, N198G	168	169[117], 155[choc]
V102N, Y203W	165	131
V102N, V197I, N198G	139[117]	n.d.
V102N	146	165[117]
V102I	121[117]	n.d.
V102L	124[117]	n.d.
S126V, P127M	179[117]	n.d.
S126F, P127N,	147[117]	n.d.
S126V, P127M, G116V, S160D	156	185
S126L, P127Q, S128A, G116V, S160D	212	187
S126M, P127A, S128G, S160D	158	143[117]
P127E	103, 130[choc]	n.d.
S130G	132	n.d.

[117]: performance measured on EMPA 117
[choc]: performance measured on CFT AS-3
n.d.: not determined

In all experiments the wash performance was determined relative to the PB92 wild type protease. In addition to the above-mentioned detergent A, the wash performance was also determined in several commercial U.S. detergents. The wash results were similar.

All publications (including patent applications) mentioned in this specification are indicative to the level of skill of those skilled in the art to which this invention pertains. All publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

CLAIM

1. A mutant protease for use in detergents which comprises:

having at least 70% homology with either the amino acid sequence of PB92 serine protease having the amino acid sequence:

H_2N-A-Q-S-V-P-W-G-I-S-R-V-Q-A-P-A-A-H-N-R-G-L-T-G-S-G-V-K-V-A-V-L-D-T-G-I-S-T-H-P-D-L-N-I-R-G-G-A-S-F-V-P-G-E-P-S-T-Q-D-G-N-G-H-G-T-H-V-A-G-T-I-A-A-L-N-N-S-I-G-V-L-G-V-A-P-N-A-E-L-Y-A-V-K-V-L-G-A-S-G-S-G-S-V-S-S-I-A-Q-G-L-E-W-A-G-N-N-G-M-H-V-A-N-L-S-L-G-S-P-S-P-S-A-T-L-E-Q-A-V-N-S-A-T-S-R-G-V-L-V-V-A-A-S-G-N-S-G-A-G-S-I-S-Y-P-A-R-Y-A-N-A-M-A-V-G-A-T-D-Q-N-N-N-R-A-S-F-S-Q-Y-G-A-G-L-D-I-V-A-P-G-V-N-V-Q-S-T-Y-P-G-S-T-Y-A-S-L-N-G-T-S-M-A-T-P-H-V-A-G-A-A-A-L-V-K-Q-K-N-P-S-W-S-N-V-Q-I-R-N-H-L-K-N-T-A-T-S-L-G-S-T-N-L-Y-G-S-G-L-V-N-A-E-A-A-T-R-COOH;

or the amino acid sequence of Subtilisin 309 serine protease having the amino acid sequence:

H_2N-A-Q-S-V-P-W-G-I-S-R-V-Q-A-P-A-A-H-N-R-G-L-T-G-S-G-V-K-V-A-V-L-D-T-G-I-S-T-H-P-D-L-N-I-R-G-G-A-S-F-V-P-G-E-P-S-T-Q-D-G-N-G-H-G-T-H-V-A-G-T-I-A-A-L-N-N-S-I-G-V-L-G-V-A-P-S-A-E-L-Y-A-V-K-V-L-G-A-S-G-S-G-S-V-S-S-I-A-Q-G-L-E-W-A-G-N-N-G-M-H-V-A-N-L-S-L-G-S-P-S-P-S-A-T-L-E-Q-A-V-N-S-A-T-S-R-G-V-L-V-V-A-A-S-G-N-S-G-A-G-S-I-S-Y-P-A-R-Y-A-N-A-M-A-V-G-A-T-D-Q-N-N-N-R-A-S-F-S-Q-Y-G-A-G-L-D-I-V-A-P-G-V-N-V-Q-S-T-Y-P-G-S-T-Y-A-S-L-N-G-T-S-M-A-T-P-H-V-A-G-A-A-A-L-V-K-Q-K-N-P-S-W-S-N-V-Q-I-R-N-H-L-K-N-T-A-T-S-L-G-S-T-N-L-Y-G-S-G-L-V-N-A-E-A-A-T-R-COOH;

differing by at least one amino acid residue at a selected site corresponding to positions 60, 87, 97, 99, 102, 116, 117, 126, 127, 128, 130, 133, 134, 154, 156, 158, 159, 160, 164, 169, 175, 180, 182, 193, 197, 198, 203, 211, and 216 in said PB92 serine protease or said Subtilisin 309 serine protease,

having improved wash performance and/or improved stability relative to said PB92 serine protease or said Subtilisin 309 serine protease.

2. A mutant protease according to claim 1, which dif-

fers from said PB92 serine protease or said Subtilisin 309 serine protease by at least one of the following mutations: [N60E], [N60E,M216S], [E87Q], [E87S], [S97D], [S99G], [S99G, V102I], [S99G,V102L], [S99G,V102N], [S99G,S130G], [S99G, Y203W], [S99G,M216S], [S99T], [V102A], [V102A,M216S], [V102E], [V102G], [V102H], [V102I], [V102I,G116V,S126V,P127M], [V102I,G116V,S126V,P127M], [V102I,S130G], [V102L], [V102L, G116V,S126V,P127M], [V102L,S130G], [V102L,M216F], [V102L, M216S], [V102M], [V102N], [V102N,XYZ, where XYZ is any modified amino acid], [V102N,R164Y], [V102N,N198G], [V102N,N198T,N198G], [V102N,N198G,Y203W], [V102N,Y203W], [V102N,L211E], [V102N,M216X, where X is any amino acid except M], [V102N,M216S], [V102P], [V102P,M216S], [V102Q], [V102Q,M216S], [V102S], [V102S,M216S], [V102T], [V102Y], [G116V,S126L,P127N, S128V,A156E], [G116V,S126L,P127N,S128V,Y203W], [G116V,S126L, P127Q,S128A,S160D], [G116V,S126L,P127Q,S128A,M216S], [G116V, S126N,P127S,S128A], [G116V,S126N,P127S,S128A,M216Q], [G116V, S126N,P127S,S128A,M216S], [G116V,S126R,P127Q,S128D,M216S], [G116V,S126R,P127Q,S128D,M216S], [G116V,S126V,P127E,S128K, S160D], [G116V,S126V,P127M,S160D], [G116V,S126V,P127M,N198G], [G116V,S126V,P127M,Y203W], [G116V,S126V,P127M,Y203G], [M117L], [S126F,P127X, where X is any amino acid except P], [S126M, P127A,S128G,S160D], [S126M,P127A,S128G,M216Q], [S126V,P127M], [P127E], [P127E,S128T,M216S], [P127E,Y203W], [P127E,L211E], [S130G], [S130G,Y203W], [L133I], [L133M], [L133W], [L133Y], [E134C], [S154D,S160G], [S154G,S160G], [S154E], [S154G], [S154N], [A156D], [A156E], [A156G], [S158D], [S158E], [S158E, I159L], [S158N], [S159E,I158L], [S160D,A166D,M169I], [S160D, N212D], [S160D,M216Q], [S160E], [S160G], [R164I], [R164M], [R164V], [R164Y], [D175E], [R180I], [V197L], [V197N], [V197T], [V197T,M216S], [V197W], [N198C], [N198D], [N198E], [N198G], [N198G,Y203W], [N198G,M216S], [N198Q], [N198S], [N198V], [Y203C], [Y203E], [Y203G], [Y203K], [Y203L], [Y203L,V193A], [Y203T], [Y203T,S182N], [Y203V], [Y203V,V193A], [Y203W], [Y203W,M216S], [L211E], [L211X,N212Z, where X is any amino acid except L and Z is any amino acid except N], [L211E,M216S], and [N212E];

3. A PB92 mutant protease according to claim 1, which

has a mutation at amino acid 102 and at least one other amino acid.

4. A PB92 mutant protease according to claim 3, which is selected from the group consisting of [S99G, V102N] and [V102N, N198G].

5. A mutant protease according to any one of claims 1 to 4 which is in substantially pure form.

6. A DNA sequence encoding a mutant protease as defined in any one of claims 1 to 4.

7. A method of preparing a mutant protease as defined in any one of claims 1 to 5, which comprises:

growing a microorganism host strain transformed with an expression vector comprising a DNA sequence encoding a mutant protease whereby said mutant protease is produced, and recovering said mutant protease.

8. A detergent additive comprising one or more mutant proteases according to any one of claims 1 to 5 and, if desired, one or more enzymes selected from the group consisting of amylases, cellulases and lipases.

9. A detergent composition comprising one or more mutant proteases according to any one of Claims 1 to 5 and, if desired, one or more enzymes selected from the group consisting of amylases, cellulases and lipases.

10. Use of a mutant protease according to any one of claims 1 to 5, in a washing process at a temperature preferably in the rage of about 15°C to about 45°C.

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/EP 93/01917

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/57 C12N9/54 C11D3/386

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 328 229 (GIST-BROCADES) 16 August 1989 cited in the application see the whole document especially page 7 lines 42-57	1-10
X	EP,A,0 405 901 (UNILEVER PLC) 2 January 1991 see the whole document	1-10
A	WO,A,89 06279 (NOVO INDUSTR) 13 July 1989 cited in the application	1-10

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 November 1993	16. 12. 93

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Van der Schaal, C

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter nal Application No
PCT/EP 93/01917

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